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Mechanisms of Immunity to Infection with Typhus Rickettsiae: Infected Fibroblasts Bear Rickettsial Antigens on Their Surfaces

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As with any immune response to infectious organisms, both antibody and T cell-mediated immune responses to infection with *Rickettsia typhi* require the appropriate presentation of rickettsial antigens to immunocompetent cells. Considering the obligate intracellular nature of rickettsiae, the exact mechanisms by which lymphocytes and macrophages encounter and respond to rickettsial antigens may depend on certain aspects of pathogenesis and on the availability of organisms or their antigens to cells of the immune system. One potential mode of rickettsial antigen presentation, not previously identified, is the appearance in vitro of rickettsial antigens on the cell membrane of *R. typhi*-infected L-929 fibroblasts. Polyvalent fluoresceinated rabbit antisera directed against whole *R. typhi* cells used in flow cytometric analysis of infected fibroblasts showed an increasing presence of *R. typhi* antigen on the host cell membrane 1 to 3 days postinfection. The significance of this finding in the pathophysiology of rickettsia-host interactions and the generation of cytotoxic T cell-mediated immunity and antibody immunity is discussed.

Immune responses to infection with typhus rickettsiae have been attributed to both antibody and cell-mediated immune mechanisms. Although the transfer of immune cells (4–6) or serum (1) can mediate resistance to rickettsial challenge, the exact immune mechanisms involved remain poorly understood. Since rickettsiae are obligate intracellular bacteria, an immune attack upon the rickettsiae while they are still inside their host cells would represent a powerful immunologic defense. To mediate such an effect, a mechanism may be involved that alerts the immune system to the presence of intracellular infection. In the present report, we show that fibroblastic tissue culture cells infected with *Rickettsia typhi* express rickettsia-specific antigens on their surfaces. Such antigens may provide triggering mechanisms for both the afferent and efferent arms of the immune system.

MATERIALS AND METHODS

Cell lines. The L-929 cell line is a fibroblastlike cell derived from a C3H/ANN mouse; the line was maintained in Eagle minimal essential medium supplemented with L-glutamine and 10% fetal calf serum, without added antibiotics.

Rickettsiae. *R. typhi* Wilmington was grown in the yolk sac of embryonated chicken eggs, purified by isopycnic banding in Renografin 76 density gradients (13), and frozen at a controlled rate to −100°C at 1 mg of protein per ml in Bovarnick sucrose phosphate glutamate supplemented with 5 mM $MgCl_2$ and 1% Renografin 76.

Infection. Tissue culture cells were infected by incubating them in 0.5 ml of Eagle minimal essential medium with freshly thawed *R. typhi* cells at a multiplicity of infection (MOI) of 3 for 1 h at 37°C, washed twice, and cultured for the indicated amount of time at 34°C in Eagle minimal essential medium containing 1% fetal calf serum and no added antibiotics. After infection, the infected or uninfected (control) cells were seeded in tissue culture flasks at a cell density of 5×10^5 cells per ml of Eagle minimal essential medium supplemented with 1% fetal calf serum and 2 mM L-glutamine. No antibiotics were added. On the day of analysis, the cells were scraped from the flasks, washed in Hanks balanced salt solution containing 0.1% fetal calf serum and 0.01% NaN_3, and stained with the reagents described below.

Fluoresceinated reagents. Rabbit antiserum to *R. typhi* (RBART) was raised by multiple injections of intact and disrupted purified rickettsia in Freund incomplete adjuvant. Antibodies were precipitated by dialysis in 18% Na_2SO_4; the precipitate was dissolved in 0.9% NaCl and dialyzed into 0.1 M $NaBO_7$ buffer (pH 9.2). Fluorescein isothiocyanate was added to give a final concentration of 20 μg/mg of protein. After stirring for 1 h at room temperature, the mixture was dialyzed back into pH 7.2 phosphate-buffered saline. As a negative control, rabbit antiserum was elicited against yolk sac subjected to the preliminary steps of the rickettsial purification procedure (RBAYS); the antiserum was purified and fluoresceinated in the same way as RBART. Antibodies were absorbed with normal mouse tissue culture cells to eliminate a slight amount of background staining on uninfected cells.

Flow cytometry. Fluorescence was measured by flow microfluorometry analysis (8) on a fluorescence-activated cell sorter (FACS II; Becton Dickinson and Co., Mountain View, Calif.) coupled to a PDP 11/40 computer (Digital Equipment Corp., Maynard, Calif.) (8). For each histogram or fluorescence profile, 10,000 viable cells were analyzed, as described elsewhere (12). After staining, the cells were fixed in 1% paraformaldehyde to inactivate the rickettsiae. This procedure has been shown to have no effect upon staining characteristics of cells analyzed on the FACS II (T. Chused, personal communication). Data are presented as fluorescence profiles, plotting cell frequency as a function of fluorescence intensity. The percentage of positive or negative cells was determined by integration of the areas under the curves.

Electron microscopy. Suspensions of L-929 cells were pelleted in microfuge tubes after being washed in saline and immediately fixed in a 1% glutaraldehyde–1% paraformaldehyde solution on ice for 30 min. After being washed three times in 0.1 M sodium cacodylate buffer (pH 7.2), the cells

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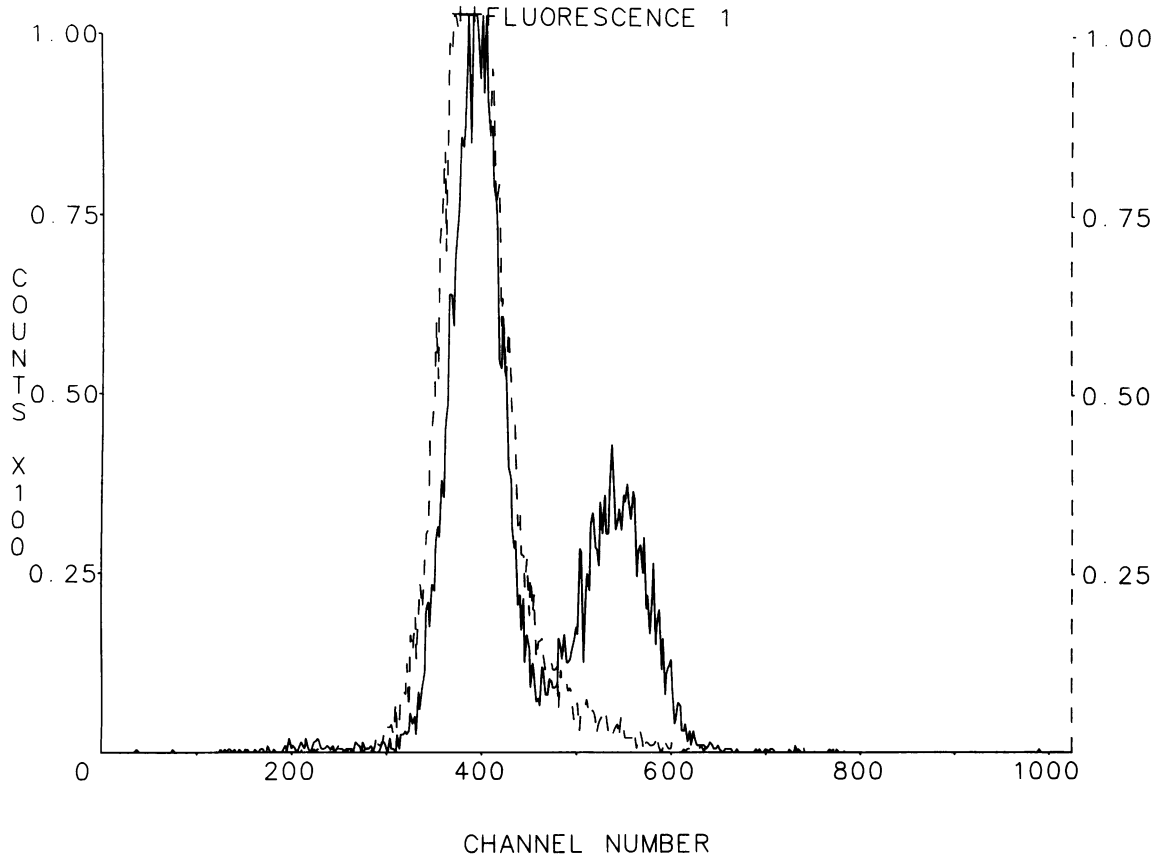

FIG. 1. L-929 cells were infected with *R. typhi* at an MOI of 3, cultured for 2 days, and stained with RBART (——) or RBAYS (– – –).

were stored at 4°C overnight in the same buffer. The samples were then postfixed in 2% OsO_4 at 4°C for 2 h and subsequently processed for electron microscopy according to standard procedures. Ultrathin sections were examined on a JEOL 100 CX transmission electron microscope.

RESULTS

Infected cells bear rickettsial antigens on their surfaces. A subpopulation of L-929 cells infected with *R. typhi* bears antigens on its surface which is detected by RBART serum (Fig. 1). The data are presented as cell frequency distribution histograms plotting cell frequency versus fluorescence intensity. The solid line represents fluorescence staining of infected L-929 cells with RBART; the dashed line represents staining with the control antiserum, RBAYS. A subpopulation of infected L-929 cells bears rickettsial antigens on its surface and is shown as the second peak at channel number 550. There is also a substantial negative population which is overlapped by the profile of RBAYS staining of infected cells. This negative peak represents the background (auto) fluorescence which is displayed to a greater or lesser extent by all cells. Light microscopic examination of these cells reveals that all cells are infected with numerous rickettsiae.

Kinetics of fluorescence acquisition. Figure 2 shows the gradual acquisition of cellular surface antigen after infection with *R. typhi*. Both after infection at an MOI of 5 (Fig. 2A) and an MOI of 25 (Fig. 2B), maximum expression of antigen is achieved after 3 days of incubation. It is interesting to note that with the higher MOI no immediate fluorescence was observed, suggesting that the fluorescence observed in Fig. 1 was not due to the deposition of rickettsial antigen at the cell surface by the entering organism.

Staining is not caused by passive adsorption of rickettsial antigen. Uninfected L-929 cells were incubated with Formalin-inactivated sonicated whole rickettsiae for 1 h at 4°C, washed twice, and then stained with RBART or RBAYS. No significant difference in staining with RBART was observed (Fig. 3A; profile of RBAYS not shown). The concentration noted with an asterisk in Fig. 3A denotes the concentration of rickettsial antigen measured by antigen-capture enzyme-linked immunosorbent assay in culture supernatant from cells infected for 3 days. In addition, uninfected L-929 cells were incubated for 3 days with supernatant from infected cultures in an attempt to ascertain whether an adherent rickettsial by-product which was present in the culture supernatant of infected cells could account for the positive staining seen in Fig. 1. The fluorescence profile shown in Fig. 3B shows the results of such an experiment. No staining was observed after the cells had been cultured with supernatant from 3-day infected cultures. This supernatant was found to contain approximately 100 to 500 ng of rickettsial antigen per ml as analyzed by antigen-capture enzyme-linked immunosorbent assay. This concentration is noted with an asterisk in Fig. 3A.

Electron microscopy. Figure 4 shows a photomicrograph of a representative L-929 cell infected with *R. typhi*. Approximately 1,000 cells were examined over the course of several experiments. Virtually 100% of the L-929 cells were infected, but none showed *R. typhi* organisms adherent to the exterior surface of the cell membrane.

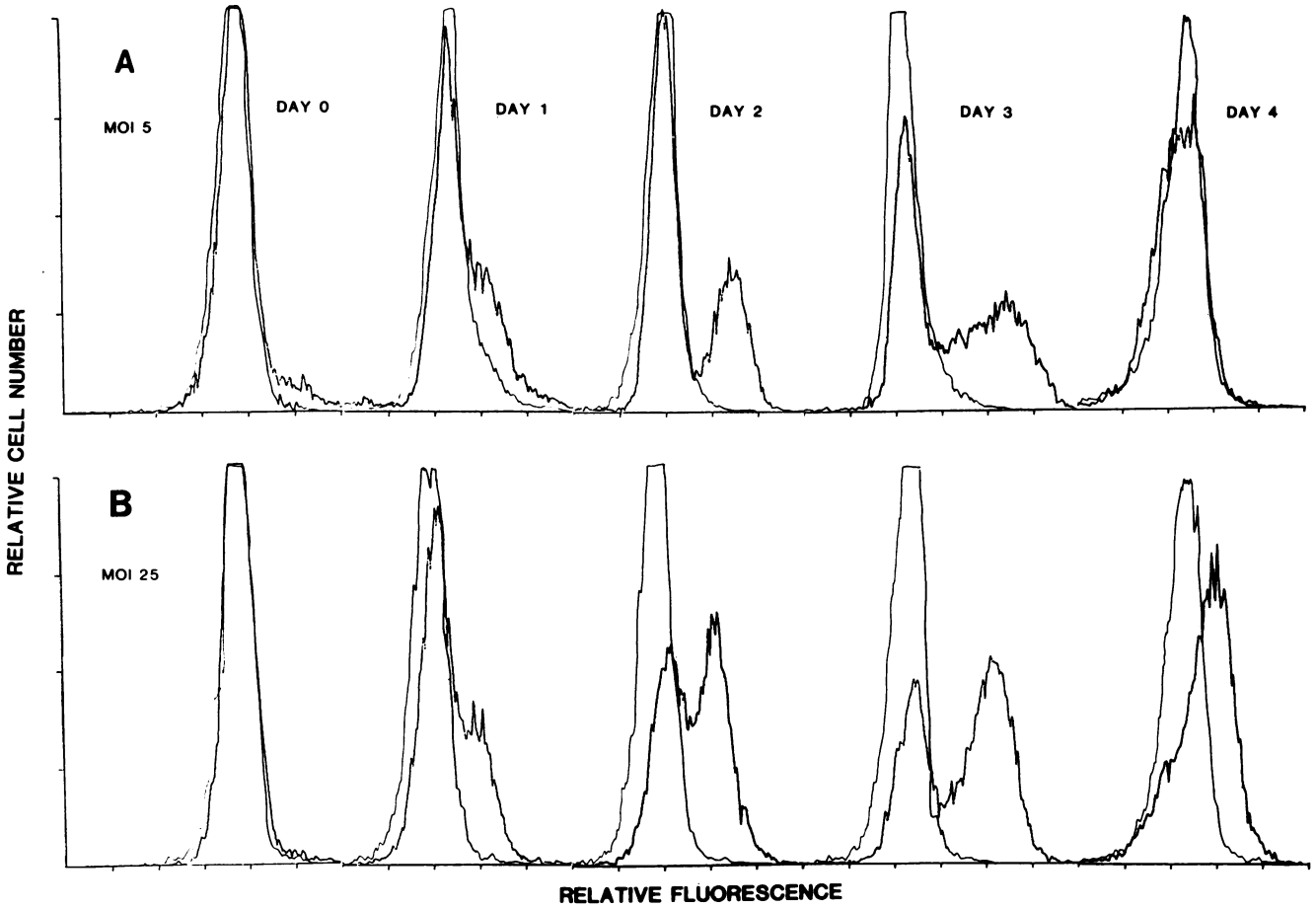

FIG. 2. L-929 cells were infected on day 0 with *R. typhi* at an MOI of either 5 (A) or 25 (B). Identical flasks were harvested at the time points shown and stained with RBART or RBAYS as in Fig. 1.

DISCUSSION

The data presented in this report show that tissue culture cells infected with *R. typhi* express rickettsial antigens on their cell surface. These antigens may be detected with an antibody directed to the rickettsiae and appear maximally after 3 days in culture.

Since rickettsiae are obligate intracellular parasites, a mechanism which would alert the immune system to the presence of infectious particles would be of survival benefit to the host. Although many other infectious agents, such as viruses (2, 11), plasmodia (3), bacteria (7), and protozoa (10), present antigens on the infected cell surface, this is the first report of a rickettsial organism presenting in such a way. In contrast with viral antigens, which are expressed in a matter of hours, the rickettsial antigen requires days for maximal surface expression. This may reflect the slower replication time of rickettsiae relative to viruses.

The simple explanation that organisms adherent to the cell surface account for the fluorescent staining observed is ruled out by the data presented in Fig. 4. In all of the electron micrographs studied in all of our experiments, we never observed a single organism adherent to the cell surface. Other simple explanations may be similarly ruled out. The data presented in Fig. 3A and B show that staining is not due to nonspecific stickiness of the infected cells. Uninfected L cells incubated with 4 μg of sonicated rickettsiae do show a slight increase in background staining; however, the pattern is completely different from the biphasic pattern in surface fluorescence staining of *R. typhi*-infected cells (Fig. 1). Although all of the cells examined are infected with numerous rickettsiae in their cytotoplasm, only a percentage of infected cells bear the relevant antigen on the surface. Dual-parameter analysis of size versus fluorescence revealed that the fluorescence-positive cells were neither small nor large but were distributed throughout the size spectrum of the cells studied. The absence of correlation with cell size suggests that the fluorescence may not be related to cell cycle. Other, preliminary experiments designed to examine intracellular bacteria in different cell lines showed either biphasic or unimodal distributions. We have not analyzed enough cell lines to make a global statement regarding fluorescence. We are currently exploring these findings in an attempt to explain this phenomenon.

R. typhi cells live in the cytoplasm of the host and multiply there, in contrast to most other intracellular bacteria, which inhabit the phagosome and prevent phagolysome formation, as in the case of *Chlamydia* spp., or multiply in the phagolysosomes, as in the case of *Coxiella* spp. (9). Since the rickettsial mRNA does not directly come into contact with the host's protein synthetic machinery, it is difficult to construct a mechanism for the findings presented in this report. However, the blood stages of malaria (3) and the intracellular protozoan *Theileria parva* (10) exhibit their

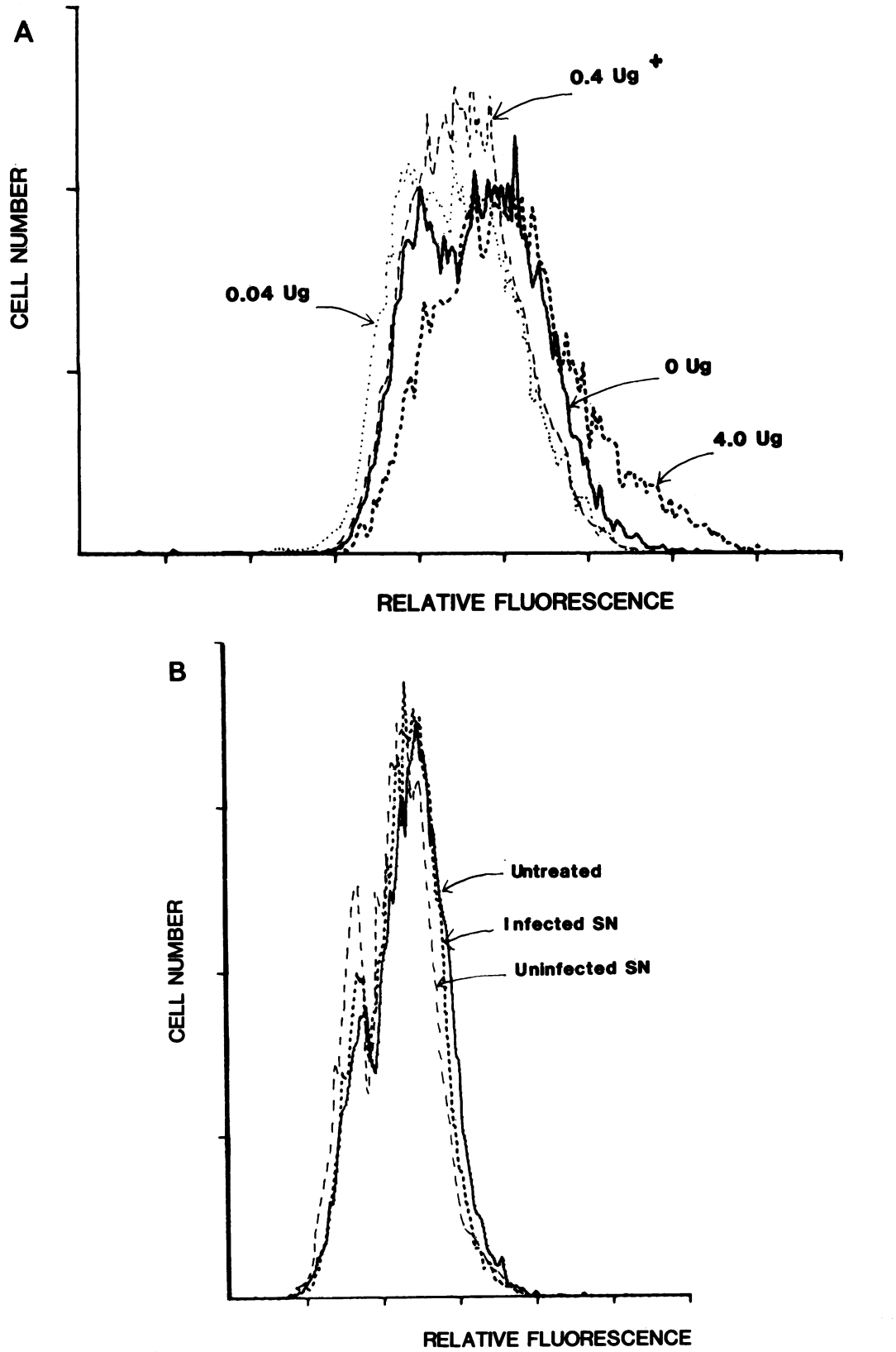

FIG. 3. L-929 cells were incubated for 1 h at 4°C with various concentrations of sonicated whole rickettsia organisms, washed, and stained with RBART. (A) The concentration marked with an asterisk (400 ng) represents the approximate amount of rickettsial antigen found in 1 ml of supernatant taken from infected L-929 cells, as in Fig. 3B. (B) L-929 cells were incubated for 3 days with supernatant from infected or uninfected tissue culture cells, washed, and stained with RBART.

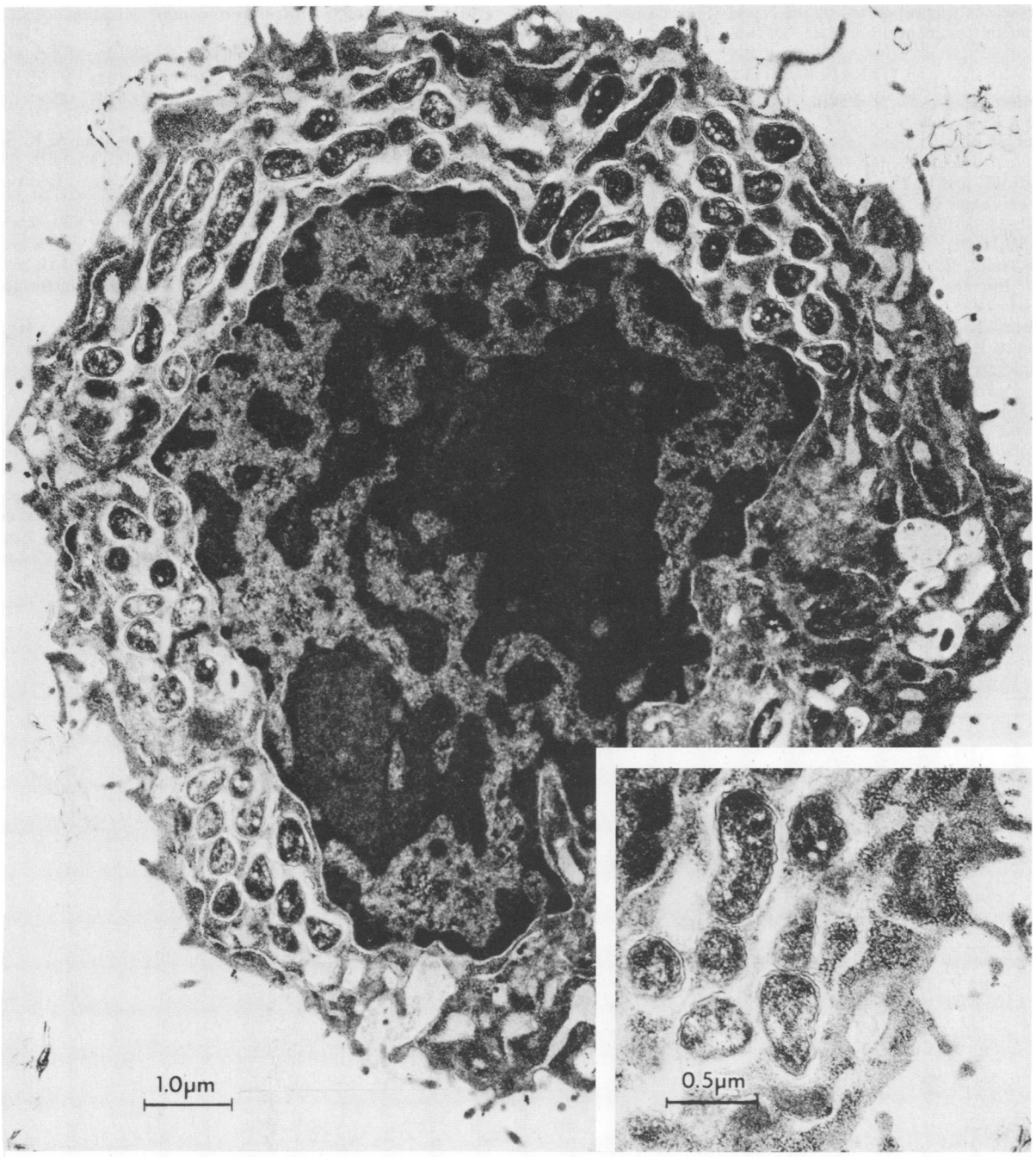

FIG. 4. Photomicrograph of L-929 cell infected with *R. typhi*. The L-929 fibroblast shows numerous round to ovoid *R. typhi* organisms crowding the cytoplasm of the cell. No rickettsiae can be seen adherent to the cell surface. Inset, Higher magnification of cell membrane.

antigens on the surface of infected cells. What is unusual about these parasites as well as rickettsiae is that they do not expose their mRNA to the cell's biosynthetic machinery as do viruses. There are fascinating biological phenomena which should be explored further.

Since intracellular bacteria and other intracellular organisms essentially are sequestered from the systemic effects of antibody and the local effects of cell-mediated immunity, a cellular mechanism which alerts the immune system to the presence of infectious particles would be of great survival benefit to the host. Rickettsiae are known to invade endothelial cells in vivo; since these cells may under some circumstances express Ia antigens on their surfaces, a possibility exists for host recognition of an Ia-rickettsial antigen complex with consequent host survival advantage.

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